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Green Butyryl-Coenzyme A Dehydrogenase

AN ENZYME–ACYL-COENZYME A COMPLEX

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1. Butyryl-CoA dehydrogenase from *Peptostreptococcus elsdenii* forms very tightly bound complexes with various acyl-CoA compounds. Spectra in some cases merely show resolution of the 450nm band, but those with acetoacetyl-, pent-2-enoyl- and 4-methylpent-2-enoyl-CoA show long-wavelength bands similar to the 710nm band of native enzyme. These complexes are formed instantaneously by the yellow form of the enzyme and much more slowly by the green form. 2. An acid extract of the green enzyme reconverts the yellow into the green form. 3. Hydroxylamine makes irreversible the otherwise reversible conversion of the green enzyme into the yellow form by phenylmercuric acetate. 4. Amino acid analysis for taurine and β-alanine shows approx. 1mol of CoA/mol of flavin in green enzyme. Anaerobic dialysis of reduced enzyme removes the CoA. On acid precipitation of green enzyme the CoA is found only in the supernatant. 5. It is concluded that native green enzyme is probably complexed with unsaturated acyl-CoA. This is shown to be consistent with findings of other workers. Catalytic activity requires displacement of the acyl-CoA, which is therefore likely to be a potent inhibitor. 6. An explanation is offered for the irreversible conversion of green into yellow enzyme by sodium dithionite. 7. The enzyme displays a feeble, previously undetected, activity towards β-hydroxybutyryl-CoA. 8. The product of oxidation of pent-4-enoyl-CoA forms a complex with reduced enzyme and strongly inhibits reoxidation of the FAD. This may contribute to inhibition of fatty acid oxidation by pent-4-enoic acid in mammals.

The flavoprotein acyl-CoA dehydrogenases of the β-oxidation pathway for fatty acids have several unusual properties. They show an exceptionally strong affinity for their substrates and products (Steyn-Parvé & Beinert, 1958*a*), and as electron acceptor they require another flavoprotein, the so-called electron-transferring flavoprotein, ETF (Crane & Beinert, 1956). An additional striking feature of butyryl-CoA dehydrogenase (EC 1.3.99.2), the dehydrogenase specific for short-chain substrates, is its bright-green colour: the enzyme from a variety of animal and bacterial sources has a broad, stable absorption band centred at about 710nm (Green, Mii, Mahler & Bock, 1954; Mahler, 1954; Steyn-Parvé & Beinert, 1958*b*; Hoskins, 1966; Engel & Massey, 1971). Mahler (1954) initially attributed the green colour to the presence of tightly bound copper, but Steyn-Parvé & Beinert (1958*b*) were able to remove all copper from the pig liver enzyme without affecting either the colour or the enzymic activity. They found that chemical reduction with sodium dithionite followed by reoxidation by air irreversibly abolished the band at 710nm, yielding a typical yellow flavoprotein with full catalytic activity. The irreversibility of this change was extremely puzzling. Mahler's (1955) report that the original spectrum of the green enzyme from ox liver returns on reoxidation has not been confirmed by subsequent work (Beinert, 1962; G. R. Drysdale, personal communication). Steyn-Parvé & Beinert (1958*b*) also found that incubation with substrates abolished the green colour, and concluded that the agent responsible for the colour was probably at the active site and interacting with the flavin prosthetic group. However, in an experiment in which the enzyme was allowed to turn over several times with substrate, the absorption at 710nm was unaffected. On the basis of this and other evidence it was concluded that the complexing agent could not be a molecule of substrate but must be a part of the protein molecule.

Several other flavoproteins have been found to form complexes with long-wavelength absorption bands on addition of compounds that are usually

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either substrates or competitive inhibitors. Long-wavelength absorption in the spectrum of a flavoprotein can frequently be ascribed to a partially reduced semiquinoid form that also gives an e.p.r.* signal. In several instances, however, there is evidence that the long-wavelength band is caused, not by semiquinone, but by a charge-transfer complex (Kosower, 1966) between flavin and another component (Palmer & Massey, 1968). Some of these complexes involve reduced flavin, but D- and L-amino acid oxidases, succinate dehydrogenase and 'old yellow enzyme' all form such complexes with the flavin in its oxidized state (Veeger, Dervartanian, Kalse, De Kok & Koster, 1966; Massey & Ganther, 1965; Matthews & Massey, 1969).

These were among the considerations that prompted a reinvestigation of the green form of butyryl-CoA dehydrogenase, now available in large amounts from a bacterial source (Engel & Massey, 1971). The realization that the purified bacterial enzyme is a variable mixture of green and yellow forms has shed new light on the problem. The present paper gives evidence that the green form of butyryl-CoA dehydrogenase is in fact a complex between the flavoprotein and an acyl-CoA, and that it is only one of a number of similar complexes that can be formed by the enzyme with various substrates.

MATERIALS AND METHODS

Butyryl-CoA dehydrogenase was purified from powdered cells of *Peptostreptococcus elsdenii* as described by Engel & Massey (1971). For experiments requiring the yellow form of the enzyme, a few grains of solid sodium dithionite were added to a solution of the green form. The reduction was done at room temperature because it is extremely slow at 2°C, and was monitored spectrophotometrically. When the absorption band at 710nm had been abolished, the enzyme was allowed to reoxidize in air, and dialysed at 2°C against 0.1M-potassium phosphate buffer, pH7.0.

Reactions with acyl thiol esters were done aerobically at room temperature in cuvettes with light-paths of 1cm and were measured with a Cary 14 scanning spectrophotometer with a Hamamatsu R-136 (S-20 response) phototube obtained from Cary Instruments, Monrovia, Calif., U.S.A. The buffer was in all cases 0.1M-potassium phosphate, pH7.0. A severalfold excess of thiol ester over enzyme was invariably used and the enzyme was 20–100μM in terms of FAD. For the purposes of the qualitative experiments described here it was only necessary to know the approximate concentrations of thiol esters. An upper limit was therefore calculated by assuming complete conversion of thiol into thiol ester. For a few experiments, in which a more accurate measure of concentration was required, the release of free thiol on treatment with NaOH was measured by using Ellman's (1959) reagent, 5,5'-dithiobis-(2-nitrobenzoic acid).

CoA (grade 1) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. (+)-Pantethine (A grade) was obtained from Calbiochem, Los Angeles, Calif., U.S.A. It was reduced to pantetheine with 2-mercaptoethanol as described by Brown & Snell (1952), freeze-dried to remove excess of reductant, dissolved in water and stored frozen. *N*-Acetylcysteamine was prepared by the method of Kass & Brock (1969).

Malonyl-CoA and 3-hydroxy-3-methylglutaryl-CoA were purchased from P–L Biochemicals, Milwaukee, Wis., U.S.A. Thiol esters of butyric acid, crotonic acid, propionic acid, acrylic acid, isobutyric acid, succinic acid and glutaric acid were prepared from the acid anhydrides (Simon & Shemin, 1953). Acetoacetyl thiol esters were prepared by the method of Lynen & Wieland (1955) as modified by Michal & Bergmeyer (1963) with diketen that had been vacuum-distilled and stored cold under N_2. β-Hydroxypropionyl-CoA was made with β-propiolactone (Vagelos & Earl, 1959). Salicyl-CoA was made as described by Tishler & Goldman (1970). All other thiol esters were made by the mixed-anhydride method of Wieland & Rueff (1953).

Most of the more common acids and anhydrides used, as well as sodium dithionite, ethyl chloroformate, oxalyl chloride and diketen were obtained from either Eastman Organic Chemicals, Rochester, N.Y., U.S.A., or Aldrich Chemicals, Cedar Knolls, N.J., U.S.A. In addition, β-propiolactone, 4-chlorobutyryl chloride, tiglic acid and senecioic acid were obtained from Aldrich Chemicals, the latter two compounds being recrystallized before use. Pent-4-enoic acid, 2-methylpentanoic acid, 3-methylpentanoic acid and hex-2-enoic acid were supplied by Pfaltz and Bauer Inc., Flushing, N.Y., U.S.A., and acrylic anhydride was obtained from K & K Laboratories Inc., Plainview, N.Y., U.S.A. Vinylacetic acid was purchased from Monomer–Polymer Laboratories (Borden Chemicals Inc., Philadelphia, Pa., U.S.A.). Pent-2-enoic acid is no longer commercially available and was therefore synthesized by a Doebner synthesis from propionaldehyde and malonic acid in pyridine with a trace of piperidine (Goldberg & Linstead, 1928). The acid was crystallized (white blades) from ice-cold ether and washed with cold ethanol. Pent-2,4-dienoic acid was similarly prepared from acrolein as described by Muskat, Becker & Lowenstein (1930). 3-Methylpent-2-enoic acid was synthesized from butan-2-one by the method of Cornforth, Cornforth, Popják & Yengoyan (1966). Hex-3-enoic acid (K & K Laboratories, but no longer available) was a gift from Dr M. J. Coon.

Rapid-reaction studies were done at 25°C with a Gibson–Milnes stopped-flow apparatus (Gibson, 1954).

Amino acid analyses as described by Engel & Massey (1971) were kindly performed for us by Dr Severino Ronchi and Dr Charles H. Williams, jun., by using a Beckman model B Analyser.

RESULTS

Formation of a stable enzyme form absorbing at 580nm. Green butyryl-CoA dehydrogenase, reduced with dithionite and allowed to reoxidize, reverts to a yellow form (Steyn-Parvé & Beinert, 1958*b*; Engel & Massey, 1971). This form, DG, was shown in the case of the pig enzyme to yield a stable

* Abbreviation: e.p.r., electron paramagnetic resonance.

complex with a perturbed flavin spectrum on treatment with the oxidized product, crotonyl-CoA (Steyn-Parvé & Beinert, 1958*b*). The same experiment done with the bacterial enzyme yielded a very similar initial result (Fig. 1*a*). The main peak in the visible spectrum was decreased in size and was shifted from 450nm to 454nm. A marked shoulder emerged at about 480nm. The bacterial enzyme (Form DG) also showed this perturbed spectrum immediately after aerobic addition of a small excess of reduced substrate, butyryl-CoA. This contrasts with the substrate-reduced pig enzyme, which is only very slowly oxidized by air over a period of days.

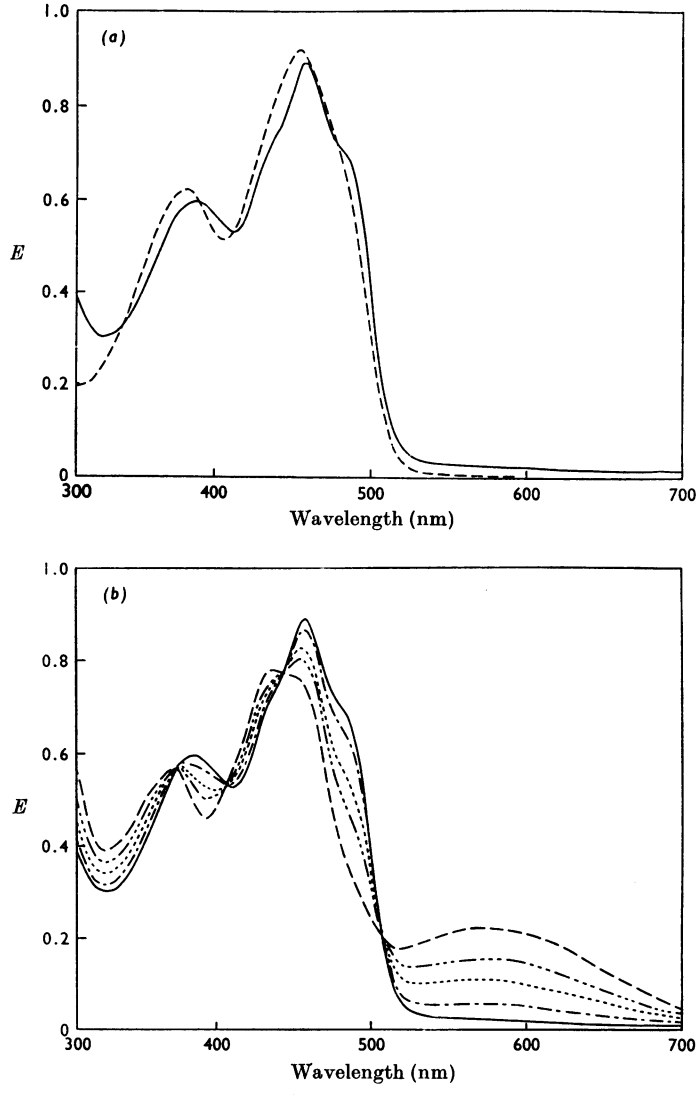


Fig. 1. Effect of crotonyl-CoA on the spectrum of the yellow form of butyryl-CoA dehydrogenase immediately after its addition (*a*) and during subsequent incubation (*b*). In (*a*), the spectrum of untreated yellow enzyme (----) is corrected for dilution. In both figures —— represents the spectrum immediately after addition of crotonyl-CoA to give a final concentration of 0.12mM. In (*b*) the subsequent spectra were obtained 10min (– – –), 27min (------), 47min (—·—·—·—) and 120min (—··—··—) after addition of crotonyl-CoA. Because the recording of each spectrum took about 6min the spectra taken within a short time of addition of crotonyl-CoA represent an approximation to the true spectrum at any given time.

When the bacterial enzyme, treated with either butyryl-CoA or crotonyl-CoA, was left at room temperature for a few hours a slow secondary change in the spectrum was observed (Fig. 1*b*). The most striking feature was the emergence of a new, broad, smooth peak at 580nm, giving the solution a greyish-green colour. There was a simultaneous blue shift in the main flavin peaks to 372 and 434nm. The 454nm peak remained only as a slight shoulder. Throughout this slow change four clear isosbestic points could be seen at 374, 404, 442 and 505nm.

The new form of the enzyme was unaltered by dialysis and its spectrum was stable over many days at 4°C. It was even more resistant than the native green enzyme to reduction by excess of dithionite: reduction, abolishing the 580nm band, took several hours at room temperature. The 580nm band, like the 710nm band of native green enzyme (Engel & Massey, 1971), was also abolished by treatment with phenylmercuric acetate. This is in contrast to the spectral perturbation immediately after addition of crotonyl-CoA to Form DG. Treatment with mercurial does not prevent the latter change.

It should be emphasized that the spectral form described here is not analogous to the partially reduced form described by Beinert (1957), which also has an absorption band between 500 and 650nm. The spectral and other similarities did suggest, however, an analogy between the 580nm- and 710nm-absorbing forms of the enzyme, and raised the possibility that the native green form might be a complex with a different acyl-CoA molecule.

Among the substances investigated in this connexion was acetoacetyl-CoA, which, written in its favoured enol form, bears an obvious resemblance to crotonyl-CoA (Scheme 1). On addition in excess to Form DG this substance immediately produced

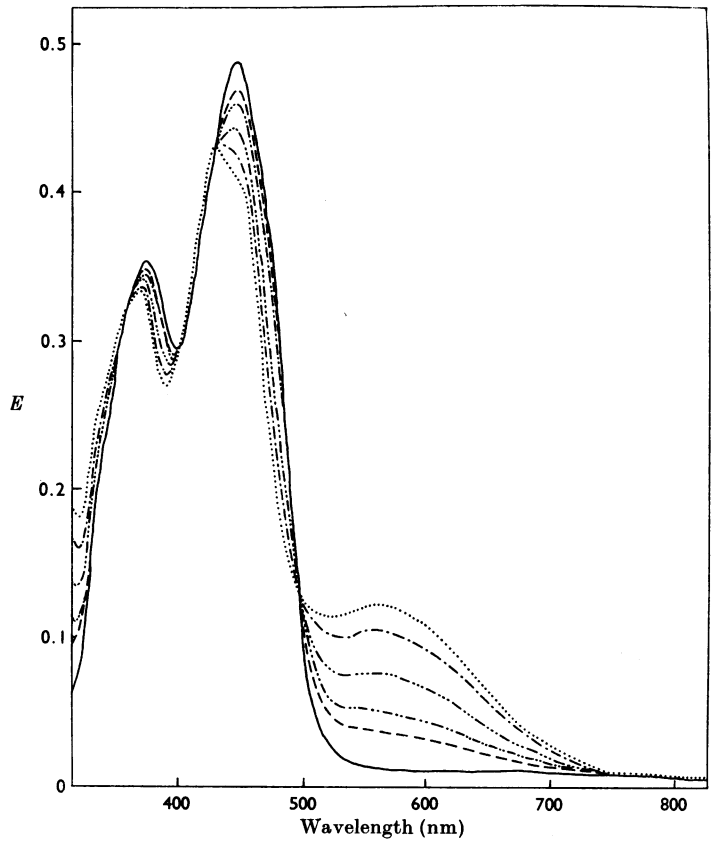


Fig. 2. Effect of acetoacetyl-CoA on the spectrum of the yellow form of butyryl-CoA dehydrogenase. Successive small volumes of 16mM-acetoacetyl-CoA were added to 0.9ml of enzyme solution. The separate curves represent total additions of 0μl (——), 2μl (– – –), 3μl (— · — · —), 5μl (— · · — · · —), 8μl (– – – –) and 26 and 36μl (· · · · · ·). The maximum addition represented 4% of the initial volume and the spectra are not corrected for this slight dilution. The addition of 36μl gave a final concentration of 0.64mM-acetoacetyl-CoA.

a large extinction in the spectrum at 580nm; the E_{580}/E_{427} ratio reached a maximum value of 0.29 in the experiment shown in Fig. 2, but values as high as 0.35 have been obtained. The positions of the peaks were identical with those found after prolonged incubation with crotonyl-CoA.

When DL-β-hydroxybutyryl-CoA was added to Form DG there was, as with butyryl-CoA or crotonyl-CoA, a slow emergence of the spectrum with the 580nm peak.

Acetoacetyl-pantetheine and acetoacetyl-*N*-acetylcysteamine both converted Form DG immediately into 580nm-absorbing forms, showing that the adenine moiety of CoA is not essential for formation of the complex. The positions of isosbestic points in these conversions are independent of the choice of thiol. Crotonyl-pantetheine, however, although it did give the initial spectral perturbation seen with crotonyl-CoA, did not produce the secondary change.

It therefore appears that the 580nm-absorbing form of the enzyme is a complex between oxidized enzyme and acetoacetyl-CoA, and that the slow changes observed with butyryl-, crotonyl- and β-hydroxybutyryl-CoA are caused by slow conversion of these compounds into acetoacetyl-CoA.

Effect of C_3 *acyl-CoA.* A parallel set of slow changes is found with the C_3 series, propionyl-, acryloyl- and β-hydroxypropionyl-CoA, although the alterations in the spectrum are less striking. The final spectrum shows merely a low band of absorption from 500 to 600nm without a peak (Fig. 3). This spectrum is presumably caused by a complex with malonic semialdehyde-CoA, although this has not been tested directly.

Transformation of native green enzyme. The native green enzyme can form the 580nm-absorbing complex without prior conversion into Form DG. The 710nm band was found to decrease as the 580nm band increased on addition of butyryl-CoA (Fig. 4), crotonyl-CoA or acetoacetyl-CoA. With butyryl-CoA and crotonyl-CoA, however, the green enzyme did not show the immediate primary perturbation of the spectrum seen with Form DG. Steyn-Parvé & Beinert (1958*b*) found that pig liver butyryl-CoA dehydrogenase was slowly 'de-greened' by substrates, although nothing corresponding to our 580nm-absorbing form was obtained.

Effect on Form DG of CoA thiol esters of other acids. A survey of small acyl-CoA compounds was undertaken to determine the specificity of complex-formation with the yellow form of butyryl-CoA dehydrogenase. Two broad categories of spectral change were observed, similar to the Type I and Type II spectral shifts described by Veeger *et al.* (1966). The thiol esters of benzoic acid, cinnamic acid and most of the unsaturated aliphatic acids used (Table 1) produced a red-shift of the spectrum,

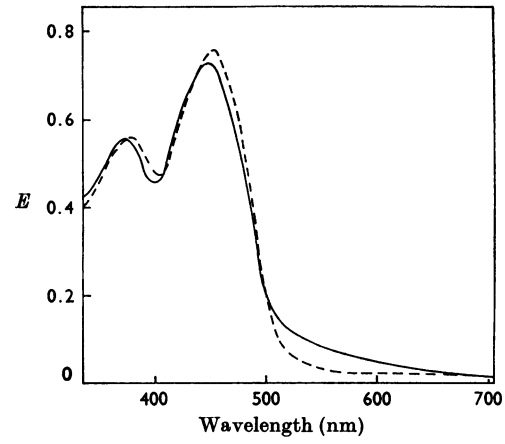


Fig. 3. Effect on the spectrum of yellow butyryl-CoA dehydrogenase of prolonged incubation with acryloyl-CoA. The spectra are those of untreated enzyme (---) and enzyme 90min after addition of a severalfold excess of acryloyl-CoA (——).

resolution of the main flavin absorption band accompanied by a decrease in its amplitude, and no long-wavelength absorption. Resolution was very marked in some cases: the C_6 unsaturated acyl-CoA compounds, for instance, shifted the 450nm peak by about 10nm and brought out a pronounced shoulder at 480–490nm. Of the remaining compounds tested, the CoA thiol esters of pent-2-enoic acid and 4-methylpent-2-enoic acid and the presumed 3-oxo acids formed complexes showing either a blue-shift of the main peak or at least a 430nm hump. All these complexes exhibited absorption bands between 500 and 1000nm, the position of the peak being very sensitive to relatively small alterations in the acyl moiety of the thiol ester. The spectra of several of these complexes were clearly similar to that of the native green enzyme, although in no case was the long-wavelength peak at 710nm obtained. Thus whereas the complex with acetoacetyl-CoA has a band centred at 580nm that with pent-2-enoyl-CoA has a peak at 810nm (Fig. 5), and that with 4-methyl-pent-2-enoyl-CoA has a peak at 900nm. The structures of these compounds are shown in Scheme 1. It is remarkable that the straight-chain C_5 $\alpha\beta$-unsaturated acyl-CoA gives a strong long-wavelength band and yet the C_4 and C_6 analogues give none whatsoever. The enzyme–pent-2-enoyl-CoA complex was taken through several cycles of reduction by sodium dithionite with complete return of the band at 810nm each time on reoxidation, in contrast with the behaviour of the native green enzyme.

The spectrum obtained with pentanoyl-CoA, like that with the corresponding C_3 and C_4 acyl-CoA

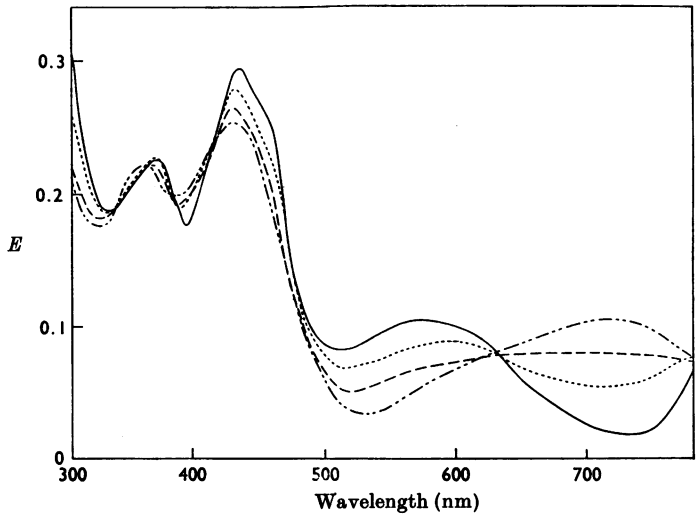


Fig. 4. Effect of prolonged incubation with butyryl-CoA on the spectrum of the green form of butyryl-CoA dehydrogenase. The curves represent spectra immediately after addition of a severalfold molar excess of butyryl-CoA (—·—·—), at 7h (– – –), 17h (·····) and 43, 51 and 67h (——).

compounds, changed with time, ultimately displaying a smooth peak at 430nm, a low peak at about 560nm and no extinction remaining at 810nm. This presumably reflects slow formation of 3-oxopentanoyl-CoA.

Prolonged incubation with vinylacetyl-CoA produced a peak at 580nm. This was also found with 4-chlorobutyryl-CoA and glutaryl-CoA, but in these two cases the result is of dubious significance, possibly reflecting impure substrate in the first case and decarboxylation of glutaconyl-CoA in the second. Among compounds found to be without effect on the spectrum of the enzyme, even when added in great excess, were malonyl-CoA, succinyl-CoA, 3-hydroxy-3-methylglutaryl-CoA and free CoA.

The results obtained with pent-4-enoyl-CoA deserve special comment. The introduction of a second double bond by the oxidation of this substrate produces a conjugated double-bond system in vinylacryloyl CoA (penta-2,4-dienoyl-CoA). When an excess of pent-4-enoyl-CoA was added to Form DG, the spectrum obtained immediately afterwards was not, as in other cases studied, that of an oxidized-enzyme–oxidized-substrate complex: extensive bleaching in the 450nm region indicated that the flavin was reduced (Fig. 6). There was, however, in the spectrum of the reduced enzyme complex a peak at 680nm, giving the solution a bluish-green colour. This complex was observed both with the normal scanning spectrophotometer in the presence of a large excess of substrate and with the Gibson–Milnes stopped-flow rapid-reaction apparatus with only a small excess of substrate. It appears that the complex of the reduced enzyme with the conjugated product is markedly stabilized towards oxygen. Reoxidation occurred over a time-scale of minutes rather than, as with butyryl-CoA, seconds. As a result reduction, as observed in the rapid-reaction apparatus, was much more extensive than that obtained with a similar excess of butyryl-CoA, although the latter substrate reduces the enzyme much more rapidly.

Effect of hydroxylamine. The above findings suggested that the native green form of the enzyme might also be a complex between the oxidized flavoprotein and an unsaturated acyl-CoA. However, the only direct evidence that the green colour might not be an inherent property of the flavoprotein itself lay in the very variable greenness of native enzyme. As a first step in testing the hypothesis of a tightly bound acyl-CoA, an agent known to react with acyl-CoA was employed, namely hydroxylamine.

Although the enzyme–acetoacetyl-CoA complex was rapidly 'de-greened' at room temperature by 0.14% neutral hydroxylamine, the native green enzyme was unaffected by ten times that concentration. Phenylmercuric acetate reversibly disrupts the 710nm-absorbing complex, and a sample of enzyme in 0.1M-potassium phosphate buffer, pH7.0 was therefore treated first with mercurial to 'de-green' it and then for 2h at room temperature with 3% (w/v) neutral hydroxylamine. Table 2 shows that, even after 2h of incubation with

Table 1. *Complexes formed between yellow butyryl-CoA dehydrogenase and various acyl-CoA compounds*

The acyl-CoA compounds were in some cases ([a]) added directly to yellow butyryl-CoA dehydrogenase. In other cases ([b]) they were produced rapidly from saturated fatty acyl-CoA compounds by aerobic turnover of the enzyme. Otherwise they were produced by prolonged aerobic incubation of the enzyme with saturated acyl-CoA ([c]), α,β-unsaturated acyl-CoA ([d]) or β-hydroxy acyl-CoA ([e]).

	Wavelength of main peak (nm)	Resolution of main peak	Long-wavelength absorption
Native green enzyme	430	–	710nm peak
Acyl-CoA added to yellow enzyme			
(a) None	450	–	None
(b) Olefinic acyl moiety			
C_3 backbone			
Acryloyl[a, b]	450	–	None
Methacryloyl[b]	450	–	None
C_4 backbone			
Crotonyl[a, b]	454	+	None
2-Methyl crotonyl[a, c]	454	+	None
3-Methyl crotonyl[a, c]	454	+	None
Vinyl acetyl[a]	454	+	None
C_5 backbone			
Pent-2-enoyl[a, b]	451	+	810nm peak
2-Methylpent-2-enoyl[b]	452	+	None
3-Methylpent-2-enoyl[a, b]	454	+	None
4-Methylpent-2-enoyl[b]	447	+	900nm peak
Penta-2,4-dienoyl[a, b]	454	+	None
C_6 backbone			
Hex-2-enoyl[a, b]	459	+	None
Hex-3-enoyl[a]	459	+	None
Hexa-2,4-dienoyl[a]	457	+	None
C_7 backbone			
Hept-2-enoyl[b]	456	+	None
(c) 3-Oxoacyl moiety			
C_3 backbone			
Malonic semialdehyde[c, d, e]	445*	–	500–650nm band
Methyl malonic semialdehyde[c]	434*	–	575nm peak
C_4 backbone			
Acetoacetyl[a, c, d, e]	434	–	580nm peak
2-Methyl acetoacetyl[d]	428	454nm hump	Low 500–600nm band
C_5 backbone			
3-Oxopentanoyl[c]	450	Large 430nm hump	560nm peak
2-Methyl-3-oxopentanoyl[c]	450	Large 430nm hump	Low 500–600nm band
(d) Other acyl moieties			
Malonyl[a]	448	–	None
3-Hydroxy-3-methylglutaryl[a]	448	–	None
Benzoyl[a]	454	+	None
Cinnamoyl[a]	454	+	None
Salicyl[a]	455	+	None

* In these two cases a new peak emerges, at 309nm in the case of malonic semialdehyde CoA and at 320nm in the case of methylmalonic semialdehyde CoA. Peaks in this region are characteristic of β-oxoacyl thioesters.

mercurial, the enzyme, when treated with excess of dithiothreitol, regained more than half the original absorption at 710nm. In contrast, the sample treated with hydroxylamine recovered only slightly on addition of the dithiol. Enzyme that had not been treated with mercurial was only slightly affected by 3% hydroxylamine.

Removal of a 're-greening' factor. If the native green enzyme is a complex similar to the enzyme–acetoacetyl-CoA complex, it should be possible to obtain from it a protein-free extract which, when added to a solution of Form DG, gives a band at 710nm.

In an experiment to test this, a solution of native

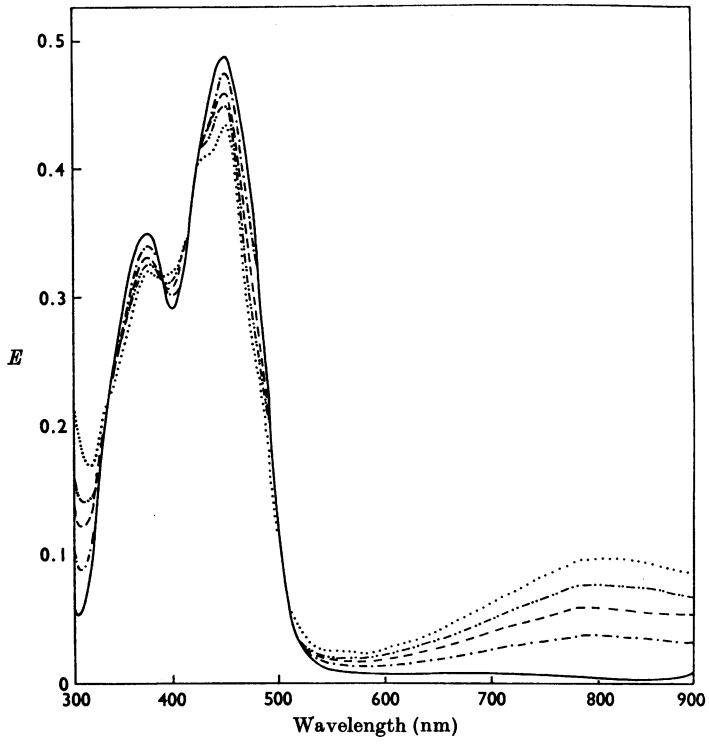
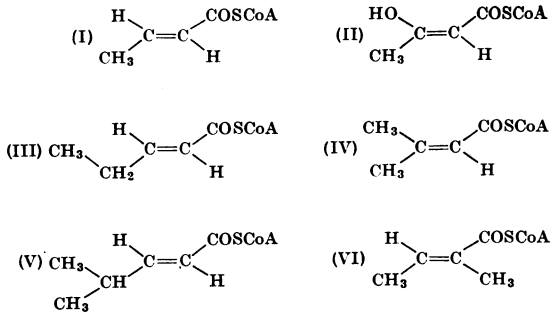


Fig. 5. Effect of pent-2-enoyl-CoA on the spectrum of the yellow form of butyryl-CoA dehydrogenase. Successive small volumes of 6.8 mM-pent-2-enoyl-CoA were added to 0.9 ml of enzyme solution. The separate curves represent total additions of 0 μl (——), 1 μl (------), 2 μl (---), 3 μl (—·—·—) and 12 and 17 μl (------). The maximum concentration of pent-2-enoyl-CoA added was 0.125 mM.



Scheme 1. Structures of some of the acyl-CoA compounds investigated. The compounds are: (I), crotonyl-CoA; (II), acetoacetyl-CoA (enol form); (III), *trans*-pent-2-enoyl-CoA; (IV), 3-methylcrotonyl-CoA (senecioyl-CoA); (V), 4-methylpent-2-enoyl-CoA; (VI), *trans*-2-methylcrotonyl-CoA (tiglyl-CoA).

enzyme was treated with 10% (w/v) trichloroacetic acid. The white precipitate was discarded and the bright-yellow supernatant was extracted several times with ether, which was finally removed in a stream of N_2. No attempt was made to remove the FAD or to concentrate the solution, as it was considered that the acyl-CoA might not be stable. When added to Form DG, the solution showed 45% of the theoretical 're-greening' capacity at 710 nm (calculated on the basis of the total absorption at 710 nm of the original green solution).

Detection of fragments of CoA. On hydrolysis the pantetheine moiety of the CoA molecule gives rise to two characteristic and unusual amino acid fragments: taurine (after oxidation) and β-alanine. These can be readily identified by amino acid analysis by the procedure of Spackman, Stein & Moore (1958): taurine is eluted very early, and β-alanine is eluted well after the other amino acids.

A complete amino acid analysis has been presented for the native green enzyme (Engel & Massey, 1971) and shows 1.02 residues of taurine and 0.80 residue of β-alanine/mol of flavin. In the present experiment a less green sample was used ($E_{710}/E_{430} = 0.27$), and 6–7 mmol (in terms of FAD) of enzyme was used for each analysis. Samples of the same batch of enzyme were subjected before analysis to various treatments as follows. Sample

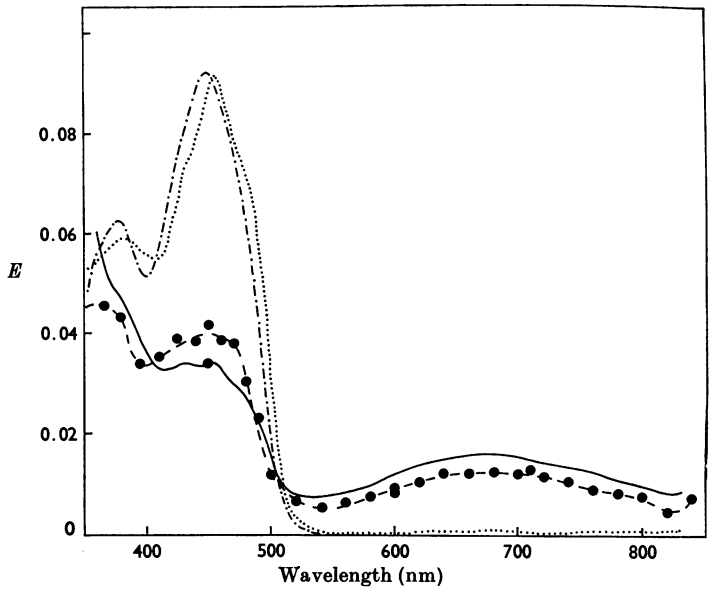


Fig. 6. Effect of penta-2,4-dienoyl-CoA on the spectrum of the yellow form of butyryl-CoA dehydrogenase. The static spectrum (------) was measured with the Cary spectrophotometer a few minutes after addition of a small excess of pent-4-enoyl-CoA (2.5 mol/mol of flavin). The separate experimental points on the line (---) represent the fullest extent of reduction achieved in the stopped-flow rapid-reaction apparatus with concentrations identical with those used in the static experiment. —— shows the reduced spectrum obtained with the Cary spectrophotometer on addition of a large excess of pent-4-enoyl-CoA (60-fold) to the yellow enzyme. All spectra including that of untreated enzyme (----) were measured at the same enzyme concentration.

1 was the control, untreated green enzyme. Sample 2 was 'de-greened' with an excess of phenylmercuric acetate. Sample 3 was 'de-greened' by reduction with sodium dithionite followed by air reoxidation. Sample 4 was also 'de-greened' by dithionite, but in this case the enzyme (1 ml) was dialysed for 12h in the reduced state against 500ml of 0.14M-potassium phosphate buffer, pH 7, previously rendered anaerobic by repeated evacuation and flushing with O_2-free N_2. The enzyme was reoxidized by dialysis against fresh aerobic buffer. Sample 5 after 'de-greening' as in sample 3 was dialysed and then treated with freshly made acetoacetyl-CoA. The total addition was four to five times that required for full development of the 580nm band. Sample 6 was treated with trichloroacetic acid at a final concentration of 10%. The yellow supernatant, after centrifugation, was withdrawn and washed with 4×2vol. of ether (sample 6a). The white pellet was triturated with 1M-K_2HPO_4, giving a white suspension that was dialysed, first against water and then against 1mM-potassium phosphate, pH 7, and centrifuged again. The supernatant was used as sample 6b and the pellet as sample 6c. All samples, except 6a, were extensively dialysed against several changes of potassium phosphate buffer, the final dialyses being against 1mM-potassium phosphate, pH 7.

The results are presented in Table 3 in terms of the ratio of taurine to aspartate plus asparagine. This ratio is 0.021 here, as compared with 0.026 for the greener sample used for the complete amino acid analysis. Treatment with mercurial, or with dithionite followed by aerobic dialysis, only lowered the taurine content slightly (0.017 and 0.018 respectively). On the other hand, anaerobic dialysis of dithionite-reduced enzyme removed 94% of the taurine. The enzyme titrated with acetoacetyl-CoA had a taurine content 2.4 times that of the original untreated sample. In the trichloroacetic acid-treated samples 73% of the taurine recovered was in the initial supernatant, sample 6a. Only a trace was detectable in sample 6c, the remaining 27% being found in sample 6b. Of the three samples, 6b and 6c showed a normal amino acid composition similar to that of sample 1. Sample 6a showed, in addition to the taurine, only glycine in significant amounts.

Table 2. *Effect of hydroxylamine on green butyryl-CoA dehydrogenase with and without prior treatment with phenylmercuric acetate*

For experimental details see the text.

Treatment		% of original E_{710} remaining	
Phenylmercuric acetate	Hydroxylamine	After treatment with phenylmercuric acetate and/or NH_2OH	2h later, immediately after addition of dithiothreitol
+	+	10	16
+	−	6	57
−	+	100	94

DISCUSSION

It has been shown above that Form DG readily gives stable complexes with the CoA thiol esters of $\alpha\beta$-unsaturated acids. In some cases the spectrum merely shows marked resolution of the absorption band in the 450nm region, but in others a new long-wavelength band appears in the spectrum, similar to the band at 710nm in the spectrum of the native green enzyme. It has proved possible to obtain from the green form by acid precipitation of the protein a supernatant fraction capable of giving rise to an absorption band at 710nm on addition to Form DG. This indicates that the agent responsible for the 710nm band is a compound of low molecular weight rather than an integral part of the protein. The finding that native enzyme gives rise to fragments of pantetheine in amino acid analysis (approx. 1 taurine and 1 β-alanine residue per FAD molecule) suggests that the complexing agent may be a tightly bound substrate or substrate analogue. After acid precipitation of the native enzyme most of the potential taurine is found in the 're-greening' supernatant fraction and none in the insoluble pellet. The reversible 'de-greening' by phenylmercuric acetate is rendered irreversible by hydroxylamine, which readily reacts with acyl-CoA compounds (Stadtman, 1957). Collectively these findings represent strong evidence that a bound acyl-CoA, probably an α,β-unsaturated or a β-oxo acyl-CoA, causes the green colour. The final proof of this clearly will rest on the isolation and identification of the acyl-CoA.

The experimental findings of Steyn-Parvé & Beinert (1958*a*,*b*) led them to the conclusion that the complexing agent was a part of the protein rather than a separate small molecule. It is therefore necessary to re-examine those findings and determine whether they are compatible with the present opposite conclusion. The following observations of Steyn-Parvé and Beinert are relevant: (i) The green and yellow forms of the pig liver enzyme are equally active in catalytic assays. (ii) Ten electrons per flavin group can be transferred from butyryl-CoA to ferricyanide in the presence of the green enzyme without any decrease in E_{710}. This was interpreted as indicating that the green enzyme is active as such, i.e. without prior conversion into the yellow form. (iii) Added radioactive substrates are bound so tightly that they can only be removed by harsh methods, such as acid treatment, which also removes the flavin, or by displacement with excess of substrate. The enzyme–substrate complexes, which in the presence of oxygen ultimately become enzyme–product abortive complexes, are fully active in catalytic assays with excess of substrate. (iv) The binding of added excess of substrate was not directly proportional to the amount added. This was taken as evidence that the enzyme as isolated did not already contain tightly-bound substrate to be displaced. With the short-chain acyl-CoA dehydrogenase this experiment was performed only with the yellow form, DG. (v) The green form bound less substrate immediately than the yellow form. At the outset of an incubation about 0.25mol of substrate/mol of flavin was bound, and, as the incubation proceeded, more labelled substrate became bound. (vi) Substrates 'de-greened' the enzyme, suggesting that the complexing agent was at the active site. This 'de-greening' was 10^4 times slower than turnover. Since even oxidized substrates such as crotonyl-CoA 'de-greened' the enzyme it was clear that the process involved displacement of the complexing agent rather than reduction of the enzyme, in contrast with 'de-greening' by sodium dithionite. (vii) The green and yellow forms were not separable by ultracentrifugation or electrophoresis, indicating only minor differences, if any, in shape, charge and molecular weight.

The greenest samples of the bacterial enzyme are considerably greener (i.e. the E_{710}/E_{430} ratio is higher) than the mammalian preparations, and yet it is clear that even these are variable but inseparable mixtures of green and yellow forms (Engel & Massey, 1971). This raises the possibility that

Table 3. *Analysis for fragments of CoA in variously treated samples of butyryl-CoA dehydrogenase*

Details of this experiment are given in the text.

Sample no.	Description	$\frac{\text{Taurine}}{\text{Aspartate}} \times 100$	Taurine (nmol/ml of original sample)
1	Native green	2.1	12.9
2	Mercurial-treated	1.7	12.0
3	Dithionite, aerobic dialysis	1.8	11.5
4	Dithionite, anaerobic dialysis	0.13	0.8
5	Titrated with acetoacetyl-CoA	5.1	28.4
6a	Trichloroacetic acid, supernatant	∞	9.9
6b	Trichloroacetic acid, redissolved pellet	0.93	3.8
6c	Trichloroacetic acid, insoluble pellet	approx. 0	approx. 0
6	Total	2.1	13.6

the pig liver enzyme studied by Steyn-Parvé & Beinert (1958*b*) was a similar mixture. The inseparability of green and DG forms [(vii) above] would allow no clear criteria of homogeneity of the green form. It is noteworthy in this connexion that in recent work (G. R. Drysdale, personal communication) the purified ox liver butyryl-CoA dehydrogenase has been much less green than that described by Mahler (1954). It is also remarkable that butyryl-CoA dehydrogenase from sheep liver has only been found so far in a yellow form (Seubert & Lynen, 1953). The assumption that the pig liver enzyme of Steyn-Parvé & Beinert (1958*b*) was not fully green makes it possible to reconcile our conclusions with their experimental observations.

If the green form is an enzyme–acyl-CoA complex, equal catalytic activity for the green and yellow forms [(i) above] is to be expected, because tightly-bound radioactive substrate can be displaced by excess of unlabelled substrate and tightly bound enzyme–substrate or enzyme–product complexes are fully active in catalytic assays [(iii) above].

The turnover experiment [(ii) above] is the central point of the argument: if a molecule of green enzyme can turn over several molecules of substrate without losing the absorption at 710nm, that absorption cannot be caused by a substrate-like molecule bound at the active site. If, on the other hand, the greenness is caused by such a substrate-like molecule, but a significant fraction of the preparation is uncomplexed yellow enzyme, the yellow component may undergo many catalytic cycles without the green component being affected or involved. Indeed it is already known [(iv) above] that 'de-greening' by substrate is 10^4 times slower than turnover. The crucial difference between the catalytic experiment, in which very dilute green enzyme performs normally, and the stoicheiometric turnover experiment, in which concentrated green enzyme apparently does not, is the presence in the former case but not the latter of a large excess of displacing substrate.

Steyn-Parvé & Beinert's (1958*b*) binding experiments [(iii) above] make it difficult to see how a mild purification procedure could yield enzyme without any tightly bound acyl-CoA as suggested [(iv) above]. In fact the native green enzyme was not examined in the experiments on proportionality of binding to substrate concentration. The finding [(v) above] that the green form bound only 0.25mol of substrate initially, but gradually more as 'de-greening' proceeded, is in excellent agreement with the hypothesis that a yellow component (25%) bound the substrate immediately whereas the green component (75%) could only do so after slow displacement of previously bound acyl-CoA.

The increase in molecular weight on binding of an acyl-CoA molecule would be only about 2%, insufficient to allow separation of the green and yellow forms on the basis of this property alone [(vii) above]. The effect on shape and surface charge cannot be predicted.

It thus appears that the present conclusion is entirely compatible with the results of Steyn-Parvé & Beinert (1958*a*,*b*).

The irreversible 'de-greening' by dithionite remains puzzling whichever hypothesis is accepted. At first sight it might even seem to eliminate the notion of a tightly bound product molecule, for reduction of such a molecule should regenerate reduced substrate. On reoxidation the enzyme should then be able to re-form its complexing agent. Explanations based on conformational or chemical changes in the protein may be discounted since Form DG can be 're-greened' at 170nm by an acid extract of green enzyme. A tentative explanation is the following: if an oxidized acyl-CoA molecule is rigidly bound at the active site, covering the flavin moiety and shielding it from the aqueous environment, a chemical reductant might replace hydrogen on the side opposite to that from which hydrogen would have been removed by the enzyme. If so, in the presence of an α- or β-substituent, chemical reduction would produce a stereoisomer incapable of being reoxidized by the enzyme. In the absence

of a substituent, switching the positions of the hydrogens would not alter the compound, and 'de-greening' should be reversible. This is precisely what is found with pent-2-enoyl-CoA. It remains to be seen whether a complexing agent with a substituent on the α- or β-carbon atom leads to irreversible 'de-greening'.

The conversion of green or yellow butyryl-CoA dehydrogenase into a stable oxidized form with an absorption peak at 580nm on prolonged incubation with butyryl-CoA requires further comment. The 580nm peak is characteristic of the enzyme–acetoacetyl-CoA complex, but it is not obvious how acetoacetyl-CoA could be produced during the incubation. The most likely answer is that the crotonyl-CoA produced immediately by the expected enzymic reaction then successively undergoes a hydration and a further oxidation. These are characteristic reactions of the β-oxidation sequence. If they occur they may be either intrinsic properties of butyryl-CoA dehydrogenase or results of contamination by other enzymes. In the case of the hydration, contamination seems the likely explanation; the enoyl CoA-hydratases that have been studied have extremely high turnover numbers (Stern, 1961) so that a minute amount of impurity could easily account for a reaction occurring over the course of several hours incubation of a concentrated solution of enzyme. Moreover, acetoacetyl-pantetheine gave rise to a 580nm band whereas crotonyl-pantetheine did not. This suggests that the specificity of a second enzyme is involved, and is consistent with the known specificity of mammalian enoyl-CoA hydratase (Stern, 1961). The formation of a 580nm band on prolonged incubation with vinylacetyl-CoA also indicates the presence of enoyl-CoA hydratase, which is known to show isomerase activity and which would therefore convert vinylacetyl-CoA into crotonyl-CoA.

The subsequent rate-limiting oxidation of β-hydroxybutyryl-CoA is most probably brought about by butyryl-CoA dehydrogenase. The enzyme that usually catalyses this reaction (EC 1.1.1.35) requires NAD. Such concentrations of nicotinamide nucleotides are unlikely to be carried through a series of purification steps involving dialysis, chromatography and precipitation and the NADH produced would in any case be readily detected in the absorption spectrum. The reaction with butyryl-CoA dehydrogenase is thermodynamically feasible, and there appears to be no *a priori* reason why substitution of one position on the β-carbon with a hydroxyl group should prevent dehydrogenation at the other position by a flavoprotein.

It will be of great interest to find out whether the mammalian butyryl-CoA dehydrogenase also forms a tightly bound complex with acetoacetyl-CoA. As might be expected, acetoacetyl-CoA is a potent inhibitor of the bacterial enzyme (P. C. Engel & V. Massey, unpublished results). Such an effect could be of considerable physiological significance.

It seems reasonable to postulate that the long-wavelength band is caused by charge-transfer between a π-donor, the unsaturated thiol ester, and a π-acceptor, FAD. However, the large shifts in absorption wavelength corresponding to small alterations in the molecular structure of the complexing agent can only be explained by invoking steric arguments. It seems, for instance, that in the series of α,β-unsaturated fatty acyl-CoA compounds the C_4 homologue is too short and the C_6 too bulky to allow the optimum interaction with the flavin that is possible in the case of pent-2-enoyl-CoA.

The experiments with pent-4-enoyl-CoA produced the first example of a fully reduced species of this enzyme displaying a long-wavelength absorption band. There are several examples in the literature of reduced-flavoprotein–oxidized-substrate complexes with long-wavelength bands: lipoyl dehydrogenase, glutathione reductase and D-amino acid oxidase all form such complexes (Massey & Palmer, 1962; Massey & Williams, 1965; Massey, Palmer, Swoboda, Williams & Sands, 1966). The striking inhibition of reoxidation after addition of pent-4-enoyl-CoA suggests that the potent hypoglycaemic action of pent-4-enoic acid in mammals, thought to be caused by inhibition of fatty acid oxidation (Bressler, Corredor & Brendel, 1969; Sherratt, 1969), may be in part caused by inhibition of butyryl-CoA dehydrogenase and sequestration of coenzyme A in the form of an enzyme–inhibitor complex. It is clearly important to repeat these experiments with enzyme from a mammalian source.

The analyses for taurine show that phenylmercuric acetate, which disrupts the green complex reversibly, does not displace the acyl-CoA completely from the surface of the enzyme. This suggests that the structures directly involved in forming the coloured complex are not the only factors responsible for tight binding. The adenosine moiety may contribute significantly to the strength of attachment of substrates and products. The high taurine content of the enzyme sample treated with acetoacetyl-CoA may reflect a certain degree of non-specific binding.

It is clear from the same series of analyses that the acyl-CoA is not bound tightly to the fully reduced enzyme. Anaerobic dialysis of chemically reduced enzyme removed nearly all the potential taurine, whereas aerobic dialysis after reduction removed little or none. This is reminiscent of the findings of Matthews & Massey (1969) with 'old yellow enzyme'. Their flavoprotein was isolated

from yeast in a green form, representing a complex of the normally yellow enzyme and an extensively conjugated small molecule. As in the present study, the small molecule is unidentified, but several related compounds form green complexes with the yellow enzyme. The position of the long-wavelength peak varies considerably among these complexes. The green forms can readily be converted into the yellow form by anaerobic dialysis of chemically reduced enzyme, as performed in the present experiments. An important difference, however, is that the complexing factors for yellow enzyme do not undergo reduction in such experiments and can be recovered from the diffusate and used to 're-green' the enzyme. This may reflect stabilization of the double-bond system by conjugation. Whereas the known enzymic function of butyryl-CoA dehydrogenase may lead to identification of the compound involved in forming the green complex, the situation is reversed for 'old yellow enzyme'. Its low NADPH oxidase activity appears to be an inadequate function and it is possible that identification of the complexing compound may lead to discovery of a substrate for this enzyme.

Succinate dehydrogenase, another flavoprotein that apparently forms charge-transfer complexes with substrate analogues, also provides an interesting parallel with butyryl-CoA dehydrogenase. Both enzymes catalyse the formation of *trans*-α,β-carbon–carbon double bonds. Whereas butyryl-CoA dehydrogenase forms a complex with acetoacetyl-CoA showing an absorption maximum at 580nm, succinate dehydrogenase has been found by Veeger *et al.* (1966) to form a complex with a peak at the same wavelength with oxaloacetate. The structural relationship between the complexing agent and the normal substrate is identical in the two cases. Veeger *et al.* (1966) speculate on the possible similarity of these complexes to the initial enzyme–substrate complex before full transfer of the reducing equivalents to the flavin.

Most striking of all, perhaps, are the numerous parallels between butyryl-CoA dehydrogenase and D-amino acid oxidase. Both enzymes have smooth, unresolved absorption bands in the 450nm region, a feature usually taken to indicate that the prosthetic group is in a hydrophilic environment (Palmer & Massey, 1968). In both cases the 450nm peak becomes resolved on addition of α,β-unsaturated compounds, acyl thiol esters with butyryl-CoA dehydrogenase and free acids with D-amino acid oxidase (Frisell, Lowe & Hellerman, 1956; Yagi & Ozawa, 1962; Massey & Ganther, 1965). In both cases partial reduction by dithionite produces a semiquinone with an e.p.r. signal, whereas partial reduction with substrate produces an absorption band in the 600nm region without an associated e.p.r. signal (Massey & Gibson, 1964; Steyn-Parvé & Beinert, 1958*b*; Beinert & Sands, 1961). Both enzymes provide examples of enzyme–product complexes showing complete bleaching in the 450nm region and considerable absorption between 600 and 700nm, butyryl-CoA dehydrogenase with penta-2,4-dienoyl-CoA (Fig. 6) and D-amino acid oxidase with imino acids (Massey *et al.* 1966). More important in the present context, both flavoproteins form stable complexes with long-wavelength absorption in the oxidized state: aminobenzoates, piperidine-2-carboxylate and pyrolline-2-carboxylate, all competitive inhibitors, form such complexes with D-amino acid oxidase (Veeger *et al.* 1966; Massey & Ganther, 1965). Finally, these two enzymes both give rise to the unusual 3,4-dihydroflavin on reduction with sodium borohydride. The only other flavoprotein that is known to do so is L-amino acid oxidase (Massey, Curti, Müller & Mayhew, 1968; Engel & Massey, 1971). The 3,4-dihydro forms of D-amino acid oxidase and butyryl-CoA dehydrogenase both exhibit a strong blue fluorescence, which is not seen with L-amino acid oxidase.

This impressive list of similarities between the two enzymes must indicate that the proteins provide very similar environments for their bound FAD. There may also be similarities in the mechanism of catalysis in the two cases and further studies of the two enzymes may prove mutually complementary.

Identification of the acyl-CoA bound to native green butyryl-CoA dehydrogenase remains an important priority. A compound that binds so tenaciously in the face of strong competition from a number of other substrates and products must be an exceedingly potent inhibitor. The presence of this complex in such diverse species as a primate, a ruminant, an omnivore and an anaerobic bacterium suggests that it may well be important in the control of fatty acid metabolism.

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